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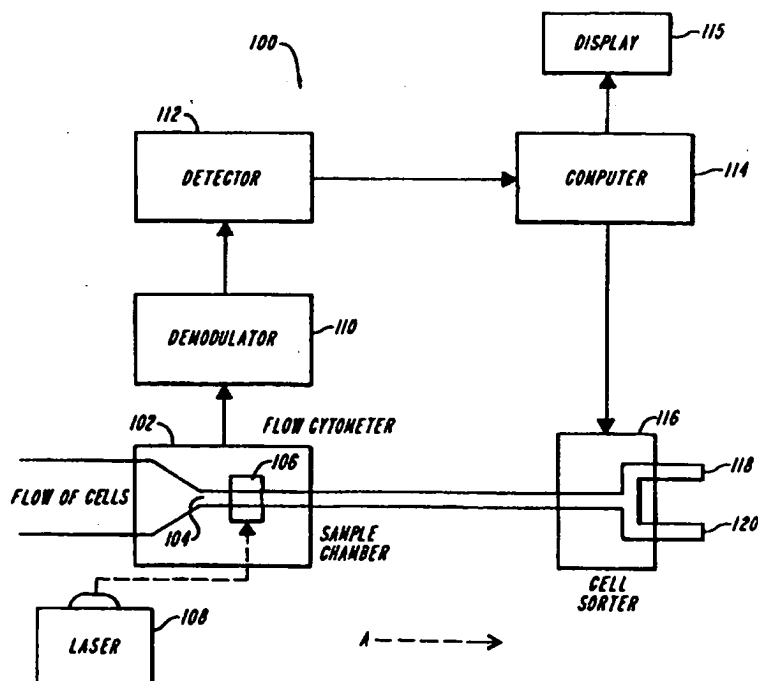
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(54) Title: SYSTEM AND METHOD FOR RAPID ANALYSIS OF CELLS USING SPECTRAL CYTOMETRY

## (57) Abstract

A system and method of detecting diseased cells in a large population of normal cells that uses flow cytometry and vibrational spectroscopy techniques. The system and method combine flow cytometry with a spectrometric system to detect inherent properties of each cell examined, inherent properties that not only discriminate between the presence or absence of disease but also the degree at which the cell is diseased. More specifically, the system and method combine the use of flow cytometry to separate cells of the sample population and Raman or resonance Raman spectroscopic techniques for detecting inherent differences in cells for the purpose of determining whether cells are diseased and level of disease in the cells. This is accomplished by forming a stream of cells by flow cytometry into the sampling region at which each cell is irradiated by an incident beam of a Raman laser. The scattered Raman spectrum from each cell is detected by a Raman spectroscopy. More specifically, a complete Raman spectrum or resonance Raman spectrum is detected for each cell flowing past the incident beam. This will provide information as to whether the specific cell in the beam of the incident light is normal or diseased, and the level of disease also may be determined on the details of the spectrum detected.



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## SYSTEM AND METHOD FOR RAPID ANALYSIS OF CELLS USING SPECTRAL CYTOMETRY

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Background of Invention

The accuracy of pathology in making medical diagnoses is generally taken for granted. However, in reality problems exist with pathology because of the inherent limitations in detecting disease by visual, microscopic examination of tissues or cells (derived from tissue). For example, one problem that exists is when this examination technique is used for diagnosing disease in cells rather than tissues. The visual, microscopic examination of cells will result in the pathologist not having many of the landmarks relied on for interpreting the presence of disease that are present with tissue. Accurate diagnosis also cannot be made when cells rather than tissues are examined microscopically because detached cells that are diseased cells are usually in such a minority to the total number of cells present that it is very easy for the diseased cells to be missed during the examination, if they are seen at all.

Advances in the ability to detect disease, through pathological examination, has depended increasingly on the examination of cells rather than tissue because cells are easier, safer, and cheaper to obtain. These advances also have looked to using samples of cells as opposed to tissues to screen for early stages of disease and especially precancer. Diagnostic pathology, as it exists, does not provide pathologists with the type of information needed for making the best-informed diagnostic and therapeutic decision mainly because of the limitations of visual, microscopy examinations.

The limitations of current diagnostic pathology are illustrated in its inability to detect residual disease in patients. Visual, microscopic examination is not able to detect leukemic cells in bone marrow samples, for example, when the concentration of leukemic cells falls below 5% of the total number of cells present. This inability to detect the leukemic cells below 5% means that for the period of time it takes for the level of diseased cells to amount to more than 5%, the physician and patient can believe he or she is in remission (when in fact that is not the case). This can cause the physician to recommend the cessation of treatment when, in fact, the treatment should continue. Therefore, it would

be desirable to have a system that provides better discrimination as to the presence of diseased cells, (for example, in bone marrow). It also is desirable to have the ability to measure accurately the number of diseased cells present when that number amounts to less than about 5% of the total cells. It is also difficult, if not impossible, to detect metastatic cancer cells in lymph nodes when the fraction of cancer cells is 5% or less of the total cells. This limited capacity of the current pathological method to provide a definitive response to treatment can have significant negative effects on the practical use of pathology.

Another serious limitation of current pathological methods is the inability to detect cancer cells in the blood of patients known to have cancer. As any clinician knows, cancer patients often have cancer cells in their blood. The presence or absence of cancer cells in blood provides an indication, after treatment, whether there is a recurrence of cancer and whether metastatic disease exists at that time. However, at present, there is no known, practical method for detecting or searching for cancer cells in blood on a routine basis.

The problem in detecting cancer cells in blood is similar to the one for detecting relatively low levels of leukemic cells in bone marrow. Cancer cells in the blood are mixed with the normal white cell elements. The white cells outnumber the cancer cells (if present) on the order of thousands of normal white blood cells to one cancer cell. The current best method of microscopic examination is to search for and detect cancer cells among a very large white blood cell fraction. To today's clinician, detecting a relatively small number of diseased cells among a far larger number of normal cells is of increasing importance in the diagnosis of disease.

The early detection of cancer cells in blood dramatically improves the chances a specific therapy will be effective. There are no suitable probes for searching for the relatively small numbers of cancer cells mixed with a very large amount of normal cells. It is known that cancer cells can be searched for in the blood using bio-chemical techniques. These diagnostic techniques require, however, that the clinician know or find an exclusive property of the cancer cells that absolutely discriminates these cells from normal cells. Moreover, current bio-chemical methods do not have the ability to discriminate cancer cells in blood in low concentrations. For example, a given probe may bind with 10,000-fold greater avidity to cancer cells versus normal cells. The number of normal cells falsely detected as cancer cells with a probe even this selective will be 1 per 10,000 normal cells

in the complete absence of cancer cells. As such, there is a guarantee that there will be false positive detection. Given these results, even a highly selective probe would not be useful for detecting cancer cells. Further, there is no way to determine what these results really mean because of the number of false negatives that also will obtain.

5           An approach to identifying a small number of abnormal cells in large mixtures of normal cells is to examine each cell in a population of interest in some rapid and automated manner. "Flow cytometry" is a technique that causes cells to flow in a stream of fluid (usually water) such that the cells line up in single file. This is accomplished by forcing the cells into tubes with diameters on the order of the diameter of a single cell. 10           Once this separation of cells into a single line is achieved, the properties of each cell can be studied, in theory, independently of the properties of any other cell.

          The examination for properties of cells separated by the flow cytometry technique generally have to be optical because of the brief period of time that each cell will be available for testing. The examination of each cell in the stream is performed by irradiation with a laser at a predetermined point in the flow path. Typically, the detection 15           system is based on the fluorescence of material in cells or the fluorescence of probe molecules attached to cells of interest. The incident beam of light excites fluorescent molecules only in the single cell being irradiated. The fluorescent signal is collected by a suitable detector, and the nature of the detection event is recorded. Scattering of incident light, based on the size of the cell in the beam, also is used as a signal to detect and measure 20           the sizes of cells.

          Natural fluorescence of normal cells and cancer cells will not discriminate between them. To provide this discrimination, attempts have been made to label populations of different cells selectively. For example, to detect small numbers of cancer cells in a large population of normal cells, there would be an attempt to label only the cancer 25           cells with a fluorescent molecule. When a tagged cancer cell passes through the beam of incident light, a signal will be detected indicating the presence of a cancer cell. When a cell passes through the incident beam and no labeling is detected, this would be categorized as a normal cell. Systems do exist for detecting and counting normal cells in the blood in this manner.

          Present day flow cytometry performs adequately for identifying rare events 30           when the fluorescent system can be constructed to collect relevant information about the

cells of interest, which depends on the ability to label the cells of interest with high selectivity. There are many ways, in theory, to selectively label cells with fluorescent molecules. One conventionally and widely used method is to mix the cell sample of interest with fluorescent antibodies that will bind specific molecular recognition sites on the surfaces of different populations of cells. Another is to make the DNA in the entire cell population fluorescent and then measure the amount of DNA per cell. The DNA content of cancer cells differs from that of normal cells. However, this difference is not always significant for the purpose of discriminating between different types of normal cells, or between cancer cells and normal cells. As such, a pathologist cannot reasonably rely upon this type of technology for finding rare cancer cells by flow cytometry. At present, there is no fluorescent method that has proved sufficiently discriminating, in the context of flow cytometry, for determining whether a given cell is a normal cell, a cancer cell, or whether a cell has properties between these extremes. This problem is magnified by the fact that diseased cells differ, even between diseased cells of the same disease. Therefore, the difficulty in finding a means to discriminate certain cell types, e.g., cancerous, is great.

There are no prior teachings of how a flow cytometer system can be combined with a detection system for discriminating between cancer and normal cells in the absence of prior treatment of the cells with labels that are specific for one or another of these types of cells at issue. In addition, the selectivity of any such labeling system is not absolute. The meaning of signals detected from probes may be uncertain because these may not label only those cells with the disease being searched for. Thus, there is a need for a rapid, easy to use, automated system for detecting diseased cells in a large population of normal cells using the flow cytometry presentation-method.

#### Summary of the Invention

The present invention is a system and method of detecting diseased cells in a large population of normal cells that uses flow cytometry, and Raman or resonance Raman spectroscopic techniques. The present invention, in essence, combines flow cytometry with a spectrometric system. This combined system does not depend on selective labeling of cells, nor the prior treatment of cells in any necessarily prescribed manner, nor the labeling of cells with probe molecules in order to discriminate between normal cells and diseased cells. To the contrary, the present invention depends, as is highly desired, on detecting inherent properties of each cell examined, inherent properties that not only

discriminate between the presence or absence of disease but also the degree to which a cell is diseased.

In addition to providing pathological diagnoses on-line, the combined system of the present invention can be used to sort cells as they exit the flow system. Cells sorted in this manner into homogenous groups then may be subjected to more extensive study of their properties.

The present invention combines the use of flow cytometry to separate cells of the sample population and Raman or resonance Raman spectroscopic techniques for detecting inherent differences in cells for the purpose of determining whether cells are diseased and level of disease in the cells. This is accomplished by forming a stream of cells by flow cytometry into the sampling region at which each cell is irradiated, for example, by an incident beam of a laser. The scattered Raman spectrum from each cell is detected by a suitable detector. More specifically, a complete Raman spectrum or resonance Raman spectrum is detected for each cell flowing past the incident beam. This will provide information as to whether the specific cell in the beam of the incident light is normal or diseased. The level of disease also may be determined.

An object of the present invention is to provide a simple, easy, and inexpensive system and method for determining the presence or absence of disease in a population of cells, and the level of disease in the cells.

Another object of the present invention is to provide a system and method that combines flow cytometry and Raman and/or resonance Raman spectroscopic techniques to determine whether a given cell is diseased, normal, or somewhere between the two.

#### Brief Description of the Drawings

Figure 1 shows a first embodiment of the system of the present invention.

Figure 2 shows a second embodiment of the system of the present invention.

#### Detailed Description of the Drawings

The present invention is a detection system that combines flow cytometry, and Raman or resonance Raman spectroscopy for detecting the presence or absence of diseased cells in a sample population of cells.

Figure 1, generally at 100, shows a first embodiment of the system of the present invention for detecting diseased cells in a large population of normal cells in the

blood, bone marrow, body fluid, tissue (treated to release cells for examination one by one in a flow system) or the like. In Figure 1, the system includes a flow cytometer 102 which causes the flow of cells in a fluid solution to separate into a single line in line 104. In line 104, the cells move one at one time in direction A.

5 Flow cytometer 104 includes sample chamber 106. The sample chamber is at a location at which each cell in line 104 is subjected to an incident beam of light from laser 108. The incident light from laser 108 is focused on each cell as it passes through sample chamber 106 of flow cytometer 102. The beam excites the cells in sample chamber 106 with light at predetermined frequencies.

10 The scattered light responses from the cell being irradiated by laser 108 are input to demodulator 110. The demodulator separates the total response into the individual frequencies representative of the scattered light of the irradiated cells in sample chamber 106. Preferably, the scattered light is collected at right angles to the incident beam that is focused on each cell so that there will be the rapid demodulation of the frequencies of the scattered light. The demodulator may be a grating, or such devices as an interferometry or  
15 tunable acoustico-optical filters. Detector 112 receives the demodulated signal and generates the vibrational spectra for scattered light and inputs it into computer 114.

Computer 114 controls the entire system. This includes flow cytometer 104, laser 108, demodulator 110, detector 112, and cell sorter 116. Computer 114, upon receiving the vibrational spectra from detector 112, compares it with stored information to  
20 determine if the cell being examined is a diseased cell or normal cell. If it is determined that it is diseased, it is also determines the level at which the cell is diseased. The results of the comparison is output to display 115 for display.

Once computer 114 makes its determination as to the type of cell that is being examined, it sends an appropriate signal to cell sorter 116 to direct the cell at issue to  
25 a particular after examination location. In Figure 1, the cells can be directed to line 118 and 120 depending on the category in which the cell is placed. This includes normal and diseased cell locations.

Raman spectroscopy is used to generate the necessary vibrational spectra for each cell because it is sensitive for detecting changes in the amount and in the chemical and physical properties of molecules in the cell, which includes both simple and complex  
30 molecules.



As the severity of disease increases in cells, it changes the chemical and physical properties of the complex molecules from which cells are built and which direct normal function. It is these discrete and often slight changes that Raman spectroscopy detects and in the case of the present invention, on a cell by cell basis. Not only are the affects of disease, at the molecular level, revealed by Raman spectroscopy, but also the  
5 affects of treatment can be determined. This helps identify which treatments are effective and which are not good treatment regimens for a certain patient.

The collection of Raman spectra in the above indicated manner for each cell has inherent advantages for diagnosis of rare events. A first is that a characteristic  
10 vibrational spectrum will not be generated for debris that contains less than the complete components of a cell. A second is that a marker of disease is an inherent property of the cell being examined and will be present if the complete components of a cell are present and the cell is diseased. A third is that the Raman spectrum provides information on the inherent properties of the cells examined and is independent of any need to label cells to make them detectable to the analytical system. A fourth is that the Raman spectrum of a cell contains  
15 a far larger amount of information about that cell than can be obtained with any current method, and, in particular, the significant differences in the spectral responses between types of cells. A fifth is that a pulsed resonance Raman laser on a rapid time scale allows for rapid screening of cells. This method also reduces the fluorescence of cells, avoids heating of the cells, and degradation of the inherent spectral information within them. A  
20 sixth is that any type of cell, even within the normal or diseased categories, can be differentiated from each other by the Raman spectrum for the cells because of the sensitivity of the response. And a seventh is that in addition to using vibrational (Raman) spectroscopy for diagnostic purposes, the spectra identified according to the present invention are usable for sorting cells.

The color of the incident beam of light may be selected to coincide with one  
25 or another molecular absorption for one of the components in cells. These components, for example, could be proteins of the cell or the nucleic acids (DNA) of the cell. According to this preferred embodiment of the present invention, the spectra obtained are resonance Raman spectra. Resonance Raman spectroscopy will produce the spectra for selected types of molecules in the cell. These spectra will not have interfering, overlapping spectral bands  
30 from other molecules in the cells. Moreover, the intensity of the scattered light in resonance

Raman spectroscopy can be as much as a million-times greater than in conventional Raman spectroscopy. This enhances signal strength and the signal-to-noise ratio of the generated signal. As such, there will be rapid collection of the spectra and rapid detection of cellular, spectral signatures for the purpose of discrimination.

5           Resonance Raman spectra can be obtained at more than a single excitation frequency. For example, the incident light can be chosen at a frequency that is absorbed by protein, e.g., 280 nm, or in a frequency absorbed by DNA. In carrying out this aspect of present invention, it is desirable to have resonance Raman spectra at more than one frequency for absorption by proteins or more than one frequency for absorption by DNA. Thereafter, it is within the scope of the present invention that the selection of frequencies  
10           for resonance Raman spectroscopy may not be limited as long as it will excite a desired cell component or components. The use of multiple frequencies will permit the selective acquisition of information via resonance Raman spectroscopy for multiple types of molecules in cells.

          When it is desired to use more than one frequency and resonance Raman  
15           spectroscopy is being used, incident beams of the desired different frequencies are arranged in tandem. The beams will irradiate cells in a predetermined sequence. The alternative method for using different frequencies to excite the cells is to arrange the excitation beams in parallel by splitting the stream of flowing cells into more than one channel. The flow of cells is to more than one sample chamber when the chambers are arranged in parallel.  
20           However, the resonance Raman spectra at different frequencies are not recorded for identical cells as in the first method.

          The parallel arrangement also may be used for collecting Raman spectra at a single excitation frequency. This arrangement allows faster throughput of cells in the system and reduces the time for examination of a single sample of cells.

25           The embodiment of the present invention that uses two parallel paths is shown in Figure 2, generally at 200. In this figure, the flow of cells of interest are input to flow cytometer 202 and flow in direction A. In flow cytometer 202, the cells enter line 204 one by one. The cell flow is split by conventional means so that the flow is divided substantially equally in lines 206 and 208. The cells in line 206 pass through sample chamber 210 where they are irradiated at a predetermined frequency by Raman laser 214.  
30           The scattered light is demodulated by demodulator 218, as described for demodulator 110,

and the demodulated signal is input to detector 220. The vibrational spectra representative of the cell is output by detector 220 and is input to computer 222 where it is compared to determine if the irradiated cell is normal or diseased, and the level of disease in the cell. Based on the results of the comparison, the computer can control cell sorter 226 to send the cell under test to either output line 230 or line 232 so that cells with the same characteristics can be placed together for further study if desired. Although only two output lines are shown, it is within the scope of the present invention that more lines can be added so that a more selective sort may take place.

The cells in line 208 undergo processing similar to the cells in line 206. Specifically, the cells in line 206 pass through sample chamber 212 where they are irradiated at a predetermined frequency (which may be the same or different from the frequency of laser 214) by laser 216. The scattered light is demodulated by demodulator 218. Detector 218 receives the output from demodulator 218 and generates a vibrational spectra representation of the cell irradiated. The spectra is input to computer 222 where it is compared and analyzed to determine if the cell under test is normal or diseased, and the level at which it is diseased if it is in fact diseased. Computer 222 controls cell sorter 228 to output the cell under test to output line 234 or line 236 depending on the type cell it is. Again, there may be more output lines if it is desirable that the sorting be more selective.

Computer 222 controls the system elements so that both of the parallel lines have access time to the computer, demodulators, detectors, and all other system elements without problem. The system also includes display 224 to display desired information.

The computer operating in real-time provides the operator with a real-time summary of the recording of events displayed on the display. The events of importance may be the number of cells counted, the number of rare events corresponding to cancer cells, the number of rare events corresponding to characteristic spectra of other known types, and the number of events not corresponding to normal events but not characteristic of cancer cells. Spectra for events already recorded can be down loaded for any purpose.

Computer analysis of spectra can be in real time. However, if desired the spectra can be collected, stored, and analyzed at a later time. It has been found that a complete spectrum of each cell will contain about 1-2 kb of data. The run time will vary depending on the sample size and testing criteria.

The system operator may select to save only a certain amount of spectra data

collected. As such, representative spectra for normal cells, for diseased cells, or for particular types of cells may be saved for a given sample. Once this is done, when the comparison is completed, the count for a particular type of cell will be incremented to show that a match was found (but the actual spectra are not saved). However, if desired, the computer can save all spectra or just spectra which do not match anything that has been saved. This also includes saving more than one type of spectrum that is categorized as diseased so that the desired grading may be accomplished.

Even given the above, the system operator may choose to store spectra of diseased cells from each examination for archival purposes along with a typical spectrum of normal cells present in the examination. This storage by the computer may be either internal or at an external location. These data also can be transmitted to a remote site or an examining laboratory. The examining laboratory may store the appropriate spectra on disks, which include identifying information about the patient and the source of the sample that was examined. In addition, the examining laboratory may prepare a personal storage device card for the patient, which contains all relevant spectral data, interpretations, basis for analysis, etc. that could be useful for following the subsequent course of the patient's disease.

The files in the storage card can be read by any facility equipped to conduct examinations according to the present invention. Therefore, there will be rapid and easy access to all relevant examinations done in the past at any time new examinations are conducted, whether or not the latter are carried out at the same facility as earlier examinations. By the data files being in the personal storage devices they will be available to the medical professional when needed to be able to compare changes in spectra of diseased cells as the patient's disease progresses or regresses.

The cells that are processed by the first or second embodiments of the present invention may be fresh. For example, the cells may be examined promptly after collection or after storage under conditions which preserve their freshness.

In addition to using fresh, untreated cells, cells fixed with a variety of chemicals known to those skilled in the arts may be processed by the present invention. The effects of fixation on the spectrum of a cell being examined are controlled by comparing the spectra of an unknown sample of cells that have been fixed prior to examination with stored data of spectra from known, well-studied, normal and diseased cells that were fixed

in the same way as the cells in the unknown sample.

The present invention provides a system and method for searching for cancer in patients suspected of having cancer but in whom careful examination by other modalities may fail to find such cancer. First, it is necessary to analyze vibrational spectra of cancer cells detected in the patient's blood. This will provide a specific diagnosis as to the tissue of origin of the cancer. This information is important for further diagnostic testing and treatment of affected patients.

Obtaining vibrational spectra of the blood of patients in whom cancer cells were found before and/or after treatment with chemical and surgical therapies for cancer will be helpful to understand the effect of the treatment regimen. For example, many patients are treated with chemotherapy after surgical removal of a breast cancer or a colon cancer. The treatment is continued for arbitrary periods and without evidence of effect in a single given patient. Therapy thus is based on the statistical effects of treatment on a large population of patients. The present invention, however, would give clear indications with regard to the effect the treatment has had.

The system and method of the present invention may also be used to identify diseases, such as leukemia. As mentioned earlier, a major problem in the treatment of leukemia is the inability to find leukemic cells in the bone marrow when the concentration of leukemic cells fall below 5%. Considering that the cancer cell burden in patients with acute myelogenous leukemia is on the order of  $10^{13}$  cells, a residual of 5% diseased cells represents  $10^{11}$  -  $10^{12}$  cancer cells present in the patient's bone marrow. The present invention will permit treatment decisions to be driven by precise knowledge of the effect specific treatments have on the numbers of cancer cells left in the patient's bone marrow at each stage during the course of treatment. The present invention will determine if a specific treatment has reached the limits of usefulness as reflected, for example, by no further decrease or increase in the numbers of cancer cells in the bone marrow.

In the case that a patient responds to a given treatment, the effect of that treatment on the spectral properties of the cancer cells becomes a marker of therapeutically positive effects. The disappearance of these markers in the spectra of cancer cells during treatment can be used as a basis for judging the efficacy of therapy in real time. This has implications for therapeutic decisions because it provides oncologists with a criterion.

about a cancer, on which to base judgments on real-time to change treatment.

The present invention may be practical for determining the onset of a relapse of disease. The sensitivity of the system and method of the present invention will determine the number of cancer cells present even though they fall into less for 5% category. This is, the present invention will determine if the number of cancer cells at levels less than 5% of marrow cells is increasing despite continued treatment.

In this same light, the present invention will allow patients in remission who are no longer being actively treated, for example, with chemotherapy, to be able to detect early any relapse by the reappearance of cancer cells in the bone marrow.

The present invention also is very useful for detecting a very small number of cells in a large fluid volume, such as cells in urine. It further includes specimens in sputum, or cells scraped from the cervix, or cells removed from solid tissue by free needles aspiration (of a suspicious nature).

The system and method of the present invention have general application to cytological examinations of any type. This present invention is favorably applied when cells in a given sample are derived from anatomically separate regions of an organ, for example, in cells in sputa that derive from all segments of the bronchial tree, or when cells in a sample are derived from multiple organs, as occurs for cells in urine (derived from kidney, bladder, collecting system of the urogenital tract, prostate) or cells in a sample removed from the cervix (cells potentially derived from the exocervix, the endocervix, uterus, and ovary.) The strength of the present invention is for cytologic examination of specimens obtained from organs (like the lung), the cervix, or urine is the ability to distinguish between each type of cell no matter the ratio of the cells present.

The present invention also may be used to detect and quantitate any type of cell with a Raman spectrum or a resonance Raman spectrum that differentiates it from other types of cells in the specimen. For example, the present invention may be used to detect selectively and to count the numbers of B and T lymphocytes in peripheral blood and to report on the presence of abnormalities in the T lymphocytes. Finally, the present invention may be used to detect evidence for infection as reflected by abnormalities/reactive changes in white cells in blood.

The terms and expressions which are used herein are used as terms of expression and not of limitation. There is no intention in the use of such terms and

expressions of excluding the equivalents of the features shown and described, or portions thereof, it being recognized that various modifications are possible in the scope of the present invention.

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**Claims:**

1. A method for detecting, characterizing and counting the cell types present in a biological sample, comprising the steps of:
  - obtaining and storing in storage means first spectra in a predetermined frequency range for each cell type that is present in such biological sample;
  - separating the cells in the biological material by flow cytometry to produce one or more single file line of cells;
  - generating second spectra one cell at a time for each single file line by passing each cell past an incident beam of light and a spectrometer means;
  - comparing the first and second spectra for variations in frequency bands for indicating the number and character of each cell type present in the biological sample; and
  - diagnosing the number and character of each cell type by the number and magnitude of variations of the first and second spectra.
2. The method according to claim 1, wherein the spectrometer means is a Raman spectroscope and the incident light is in the visible, ultraviolet, near infrared or infrared region of the light spectra.
3. The method according to claim 1, wherein the incident light and spectrometer means are appropriate for resonance Raman spectroscopy.
4. The method according to claim 1, wherein the incident light and spectrometer means are appropriate for near infrared absorption spectroscopy.
5. The method according to claim 1, wherein the incident light and spectrometer means are appropriate for differentiating cells from each other on the basis of their Raman, resonance Raman, and/or combinations of resonance Raman spectral data obtained at more than one frequency of incident light.
6. The method according to claim 1, wherein the biological sample is bone marrow, blood, sputa, urine, ascitic fluid, cerebral spinal fluid, pleural fluid, cytological specimens acquired by fine needle aspiration of tissues, cervical tissue, uterine tissue, in any other sample containing live or chemically fixed cells that have been dissociated from each other.
7. A method for diagnosing cancer by detecting low levels of cancer cells in a patient's blood or bone marrow containing normal cell types, comprising the steps of:
  - obtaining and storing in storage means first spectra in a predetermined frequency



range for each normal cell type that is present in such patient's blood or bone marrow;

separating the cells in the patient's blood or bone marrow by flow cytometry to produce one or more single file line of cells;

generating second spectra one cell at a time for each single file line by passing each cell past an incident beam of light and a spectrometer means;

comparing the first and second spectra for variations in frequency bands for indicating the number of cancer cells present in the patient's blood or bone marrow;

diagnosing the number of cancer cells present by the number and magnitude of variations of the first and second spectra.

8. The method according to claim 7, wherein the cancer is residual leukemia.

9. A method for evaluating the course of cancer by carrying out the method according to claim 7 at a first time point and at a second later time point and comparing the number of cancer cells present in the patient's blood at each time point.

10. A method for evaluating a response to cancer therapy by carrying out the method according to claim 9, wherein the first time point is before administration of therapy and the second time point is after administration of therapy.

11. A method for evaluating changes in biological properties or phenotypic properties of cancer cells over time in a patient's blood, comprising the steps of:

obtaining and storing in storage means first spectra in a predetermined frequency range for normal cells and cancer cells that are present in such patient's blood at a first point in time;

separating the cells in the patient's blood by flow cytometry to produce one or more single file line of cells;

generating second spectra at a second later point in time one cell at a time for each single file line by passing each cell past an incident beam of light and a spectrometer means;

comparing the first and second spectra for variations in frequency bands for indicating biological or phenotypic changes in cancer cells present in the patient's blood;

diagnosing the biological or phenotypic changes of cancer cells present by the number and magnitude of variations of the first and second spectra.

12. A method for evaluating a response to cancer therapy by carrying out the method according to claim 11, wherein the first time point is before administration of therapy and the second time point is after administration of therapy.

13. A method for detecting and typing bacteria possible present in body fluids, comprising the steps of:

obtaining and storing in storage means first spectra in a predetermined frequency range for each cell type that is present in such body fluid and for each bacterial type that is possibly present in such body fluids;

separating the cells in the biological material by flow cytometry to produce one or more single file line of cells;

generating second spectra one cell at a time for each single file line by passing each cell past an incident beam of light and a spectrometer means;

comparing the first and second spectra for variations in frequency bands for indicating the number and type of bacteria present in the biological sample;

diagnosing the number and type of bacteria present by the number and magnitude of variations of the first and second spectra.

14. A method for assessing sepsis by detecting changes in leukocytes in a patient's blood, comprising the steps of:

obtaining and storing in storage means first spectra in a predetermined frequency range for unactivated and activated leukocytes;

separating the cells in the patient's blood by flow cytometry to produce one or more single file line of cells;

generating second spectra one cell at a time for each single file line by passing each cell past an incident beam of light and a spectrometer means;

comparing the first and second spectra for variations in frequency bands for indicating the number of activated leukocytes cells present in the patient's blood;

diagnosing whether sepsis is present based upon the number of activated leukocytes present as determined by the number and magnitude of variations of the first and second spectra.

15. The method according to claim 14, wherein the leukocytes are T cells.

16. The method according to claim 14, wherein the leukocytes are B cells.

17. A system for detecting and characterizing the type of cells present in a biological sample, comprising:

a flow path suitable for the passage of one cell at a time in a predetermined direction;

a sample chamber disposed across the flow path at a predetermined location;  
a light source capable of irradiating a single cell located at the sample chamber so as to excite at least one component of the cell a predetermined frequency to generate a spectrum based on the excited cell;

a detector for detecting the generated spectrum and processing the spectrum to generate vibrational spectrum; and

signal processing means for receiving the vibrational spectrum and comparing it with stored information to determine whether the irradiated cell is diseased or normal.

18. The system according to claim 17, wherein the light source includes a laser for exciting at least a Raman spectrum.

19. The system according to claim 17, wherein the light source includes a laser for exciting at least a resonance Raman spectrum.

20. The system according to claim 17, wherein the system further includes a cell sorter for sorting cells output from the flow path according to predetermined characteristics.

21. A system for detecting and characterizing the type of cells present in biological sample, comprising:

a flow path that has first and second parallel branches with the first and second branches each being suitable for the passage of one cell at a time in a first direction;

a first sample chamber disposed across the first branch of the flow path at a predetermined location;

a first light source capable of irradiating a single cell located at the first sample chamber so as to excite at least one component of the cell at a first predetermined frequency to generate a first spectrum based on the excited cell;

a second sample chamber disposed across the second branch of the flow path at a predetermined location;

a second light source capable of irradiating a single cell located at the second sample chamber so as to excite at least one component of the cell at a second predetermined frequency to generate a second spectrum based on the excited cell;

a detector for detecting the first and second spectrum and processing the first and second spectrum to generate a first and second vibrational spectrum;

signal processing means for receiving the first and second vibrational spectrum and

comparing therein with stored information to determine whether the irradiated cells are diseased or normal.

22. The system according to claim 21, wherein the first light source includes a laser for exciting at least a Raman spectrum.

23. The system according to claim 21, wherein the first light source includes a laser for exciting at least a resonance Raman spectrum.

24. The system according to claim 21, wherein the system further includes a cell first sorter for sorting cells output from the first branch of the flow path according to predetermined characteristics.

25. The system according to claim 21, wherein the second light source includes a laser for exciting at least a Raman spectrum.

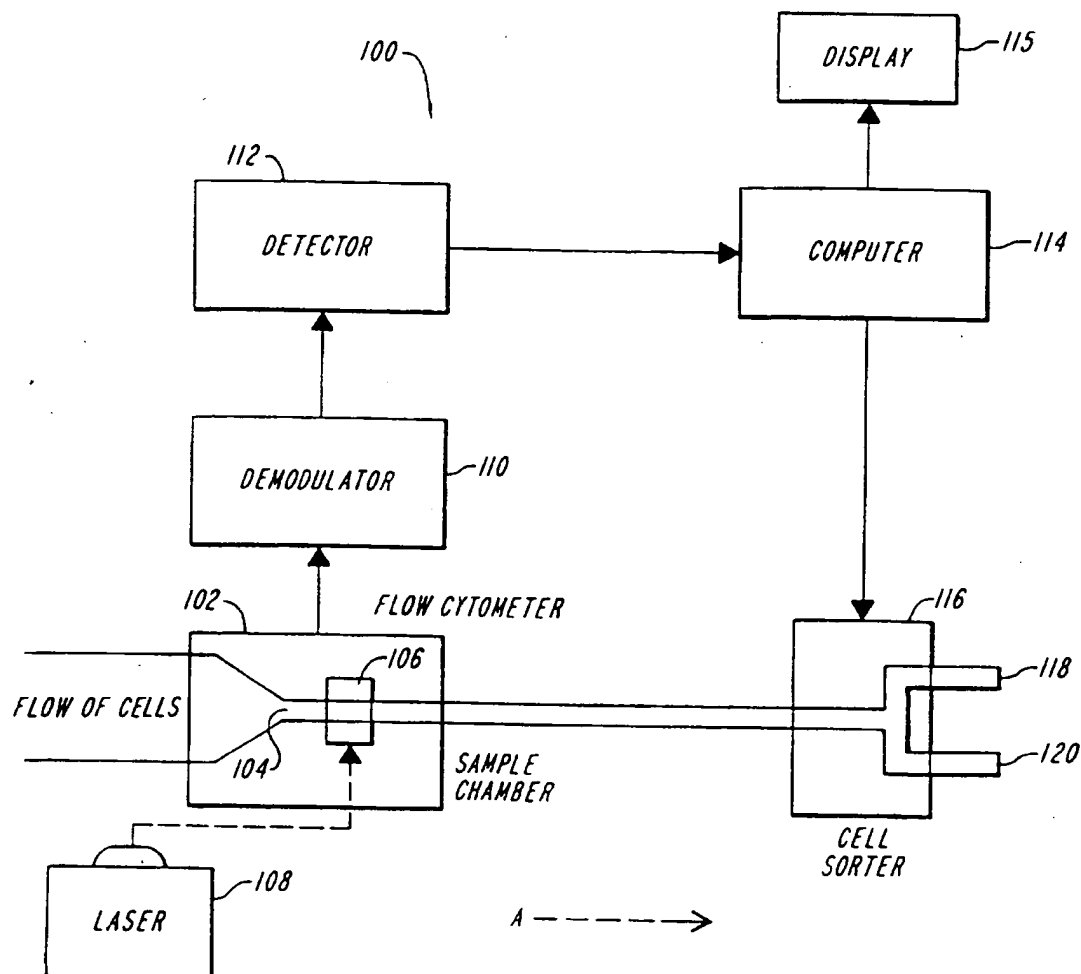
26. The system according to claim 21, wherein the second light source includes a laser for exciting at least a resonance Raman spectrum.

27. The system according to claim 21, wherein the system further includes a second cell sorter for sorting cells output from the second branch of the flow path according to predetermined characteristics.

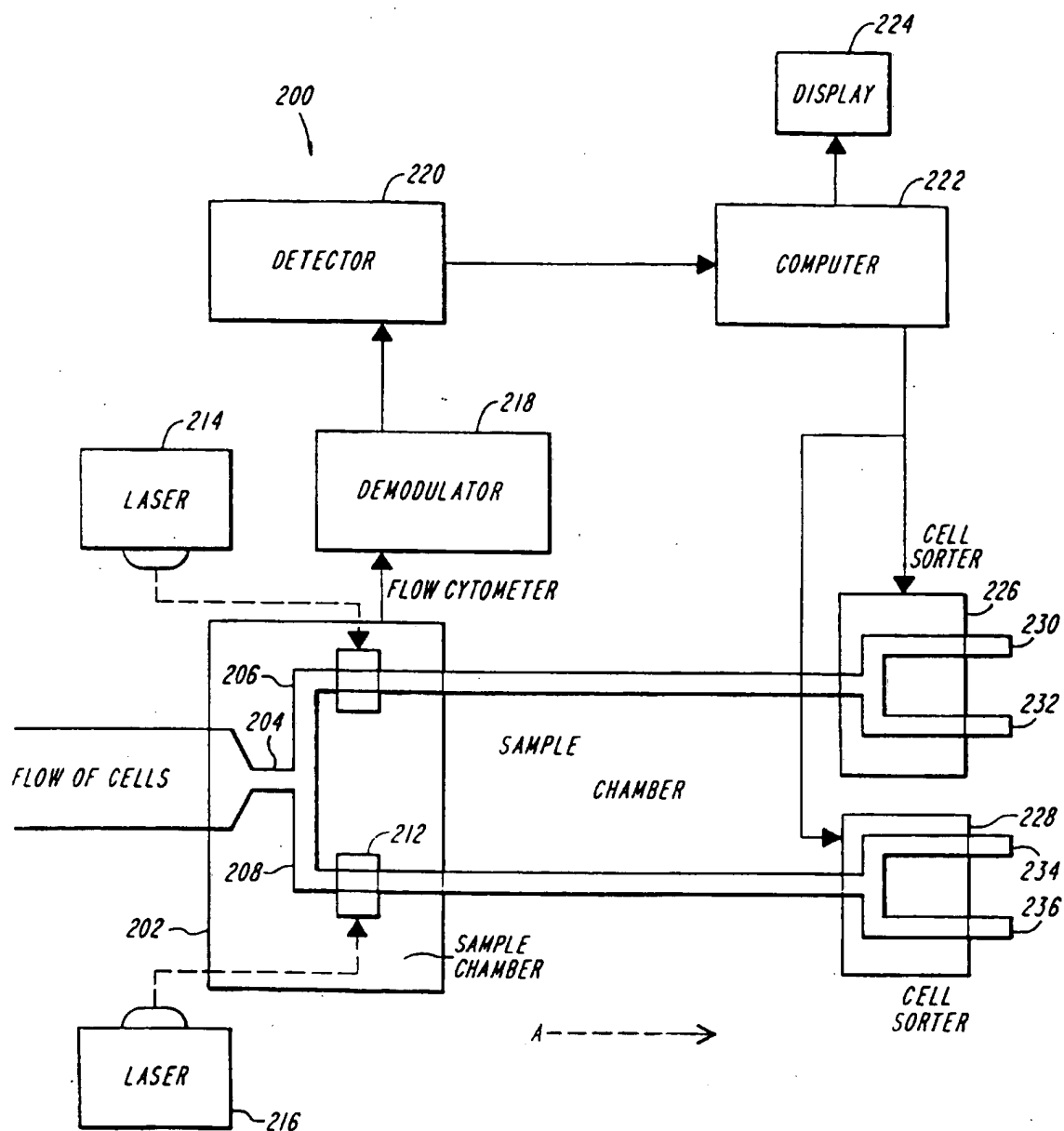
28. The system according to claim 21, wherein the first and second predetermined frequencies are the same.

29. The system according to claim 21, wherein the first and second predetermined frequencies are different.

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**FIG. 1**

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**FIG. 2**

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/01625

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 G01N15/14

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 08120 A (MACQUARIE UNIVERSITY) 14 May 1992 see abstract	1, 7, 17
A	see page 1, line 6 - line 17 see page 6, line 14 - line 34 see page 7, line 1 - line 2	21
Y	see figures 4-6	2, 3, 13, 18, 19
Y	--- WO 93 07471 A (BECKMAN INSTRUMENTS) 15 April 1993 see page 1, line 9 - page 2, line 9 see page 2, line 29 - page 3, line 4 see page 3, line 23 - line 33 see page 8, line 15 - line 35 see page 12, line 19 - page 13, line 3 see page 13, line 17 - page 14, line 7	2, 18
A	see figures 1, 6 --- -/-	22, 25

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- "&" document member of the same patent family

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category <sup>a</sup>	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 847 198 A (NELSON) 11 July 1989 see abstract see column 5, line 7 - line 10 see column 5, line 49 - line 57	3,13,19
A	see column 5, line 65 - column 6, line 2 ---	22-26
Y	US 5 483 469 A (VAN DEN ENGH) 9 January 1996 see abstract see column 7, paragraph 2; figure 2 ---	21,24
Y	WO 94 29712 A (UNIVERSITY OF ALBERTA) 22 December 1994 see page 1, line 14 - line 18 see page 5, line 21 - line 35 see page 22, line 26 - line 35 see page 23, line 15 - line 31 see figure 1 -----	21,24



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/01625

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9208120 A	14-05-92	NONE	
WO 9307471 A	15-04-93	US 5292483 A AU 2761392 A EP 0539743 A JP 5209832 A US 5484571 A	08-03-94 03-05-93 05-05-93 20-08-93 16-01-96
US 4847198 A	11-07-89	NONE	
US 5483469 A	09-01-96	NONE	
WO 9429712 A	22-12-94	US 5439578 A AU 6922394 A CA 2164207 A EP 0701694 A US 5584982 A	08-08-95 03-01-95 22-12-94 20-03-96 17-12-96